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APPLICATION FOR LETTERS PATENT

for

EFFICIENT EXPRESSION OF PLASMODIUM APICAL MEMBRANE ANTIGEN IN YEAST CELLS

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Title of Invention:

EFFICIENT EXPRESSION OF PLASMODIUM APICAL MEMBRANE ANTIGEN IN YEAST CELLS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application is a continuation of PCT International Patent Application No. PCT/NL/01/00934, filed on December 21, 2001, designating the United States of America, and published, in English, as PCT International Publication No. WO 02/052014 A2 on July 4, 2002, the contents of the entirety of which is incorporated by this reference.

TECHNICAL FIELD

The invention relates to the fields of medicine, vaccines and diagnostics. More in particular the invention relates to the intervention with and the diagnosis of Plasmodium induced malaria.

BACKGROUND

Malaria is a wide-spread disease in most (sub)tropical countries. It is acquired by infection with a malaria parasite. The socioeconomic impact of this disease is enormous. Malaria exists in different forms, caused by different parasites. The symptoms vary considerably between the different forms.

Plasmodium vivax and Plasmodium falciparum are the two most important human malaria parasites. Other human malaria parasites are Plasmodium ovale and Plasmodium malariae, but these two species are less pathogenic than P. vivax and P. falciparum. P. vivax causes less mortality than P. falciparum. Treatment of P. falciparum is becoming more complicated, because chloroquine resistant P. falciparum parasites are spreading rapidly and multidrug resistant parasites have also developed. In addition, chloroquine resistant P. vivax has been detected, indicating similar problems in treatment of P. vivax as for P. falciparum.

At present, there is essentially no effective vaccine available against malaria, at least not for use in humans. Accumulated data, including that from non-human primate [1] [2] and rodent studies, [3] [4] have indicated that the apical membrane antigen-1 (AMA-1) family of molecules is a target for protective immune responses. In all Plasmodium species reported to date, with the exception of Plasmodium falciparum [5] and P. reichenowi [6] that form a phylogenetic clade distinct from other malaria parasites, AMA-1 is synthesized de novo as a 66 kDa transmembrane protein. The protein contains a predicted N-terminal signal sequence, an ectodomain, a predicted transmembrane region and a C-terminal cytoplasmic domain. The ectodomain is further divided into

three domains (domain I, II and III) defined by disulfide bonds [7]. In *P. falciparum* and *P. reichenowi* the protein is expressed as an 83 kDa protein having an N-terminal extension as compared to the 66 kDa forms, referred to as the prosequence. Intra-species sequence polymorphism due to point mutations [8] [9] [10] reveals clustering of mutations in particular domains of the molecule. Despite this, between species there is considerable conservation of primary amino acid structure and predicted secondary structure. Evidence to date indicates that protection invoked by AMA-1 is directed at conformational epitopes [1] [3] [4] [11] located in the AMA-1 ectodomain. Immunisation with reduced AMA-1 fails to induce parasite inhibitory antibodies [3] [11] and so far only those monoclonal antibodies that recognize reduction-sensitive conformational AMA-1 epitopes have been shown to inhibit parasite multiplication *in vitro* for *P. knowlesi* [12] [13] and *P. falciparum* [6] [14]. This indicates that for an AMA-1 vaccine the correct conformation will be critical.

Recombinant expression of *P. falciparum* AMA-1 (Pf AMA-1) in a conformational relevant way that allows production of clinical grade material has been notoriously difficult. One characteristic important for recombinant expression techniques is the unusually high A+T content of *P. falciparum* codons in comparison to most other organisms and in particular in comparison to most other organisms generally used for recombinant protein expression. The group of Prof. Anders (WEHI, Australia) has developed expression of the ectodomain in *E. coli*, followed by a refolding protocol, but scaling up this process to levels that allow production of clinical grade material has proven cumbersome. Because eukaryotic expression systems are likely to produce material with the correct disulphide bonds directly, we have focused upon expression in such systems. Expression of the full length 622 amino acids long Pf AMA-1 protein (7G8 strain) in insect cells using recombinant baculovirus resulted in expression on the surface of insect cells [15]. The protein migrated in SDS-PAGE more slowly than the native molecule indicating glycosylation.

Expression in the presence of tunicamycin confirmed this. Said Pf AMA-1 protein was used to raise rat monoclonal antibodies (mAbs), some of which could block parasite multiplication in an *in vitro* assay. These functional mAbs recognised a conformational epitope located in the ectodomain of Pf AMA-1. Reactivity with these mAbs, especially with mAb 4G2, is used as one assay for proper folding of recombinant Pf AMA-1. Relatively low expression levels did not allow the baculovirus sytem to be developed for the production of clinical grade material.

We have obtained high level expression of *P. vivax* AMA-1 (Pv AMA-1) ectodomain in the methylotrophic yeast *Pichia pastoris* [16]. However, this expression system is not likewise suitable to produce a secreted ectodomain of Pf AMA-1. Using the same expression vector as has successfully been used for Pv AMA-1, recombinant Pf AMA-1 *P. pastoris* clones do not express Pf AMA-1 ectodomain at any level. Analysis of total RNA extracted from induced cultures revealed only truncated mRNA products for Pf AMA-1. So no effective expression of Pf AMA-1 was possible until the present invention. This was a problem because expression of homogeneous Pf AMA-1 in high amounts is highly desirable. Efficient production of Pf AMA-1 gives possibilities to develop a diagnostics, or a vaccine and/or a medicine against *P. falciparum* and/or other *Plasmodium* species. Presently, such a vaccine or medicine is not available.

The present invention provides a method for producing mRNA encoding *Plasmodium* AMA-1 ectodomain, or a functional part, derivative and/or analogue thereof, in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize said yeast's codon usage. Preferably, said ectodomain is derived from a 83 kDa AMA-1 protein. Particularly the ectodomain of 83 kDa AMA-1 proteins are difficult to express in yeast cells. More preferably, said 83 kDa AMA-1 protein

is derived from *Plasmodium falciparum*. Now that a method of the invention is available, it is also possible to produce an analogous protein, such as a complete AMA-1 protein. Thus the invention also provides a method for producing mRNA encoding *Plasmodium* AMA-1 protein, or a functional part, derivative and/or analogue thereof, in a yeast cell, comprising providing said yeast cell with a nucleic acid encoding said *Plasmodium* AMA-1 protein, said nucleic acid being modified to utilize said yeast's codon usage. AMA-1 ectodomain produced with a method of the invention comprises at least one conformational epitope that is comparable to a conformational epitope in native AMA-1 ectodomain, produced by the parasite, preferably in a human host. AMA-1 ectodomain of the invention can be used for vaccination purposes and for diagnostic purposes.

With a method of the invention, it is possible to obtain mRNA encoding AMA-1 ectodomain in a yeast cell. In said yeast cell, said mRNA is efficiently translated into a functional AMA-1 ectodomain. With the teachings of the invention, a person skilled in the art is able to produce a functional part, derivative and/or analogue of said ectodomain comprising at least one immunogenic property of native ectodomain in kind not necessarily in amount.

In a preferred embodiment a method of the invention further comprises allowing for expression of said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof in said yeast cell. Preferably, said AMA-1 ectodomain or functional part, derivative and/or analogue thereof is purified from said yeast cell and/or culture medium.

By a *Plasmodium* AMA-1 ectodomain is meant herein a part of a *Plasmodium* AMA-1 protein which is normally present between the N-terminal signal sequence and the transmembrane region of a naturally occurring *Plasmodium* AMA-1 protein. In *Plasmodium falciparum*, said ectodomain normally spans amino acid residues 25 to 545. In a preferred embodiment an ectodomain of the invention spans an amino acid sequence corresponding to amino acid residues 25 to 545 in *Plasmodium falciparum*.

A functional part of a *Plasmodium* AMA-1 ectodomain is defined herein as a part which comprises at least one immunogenic property of said AMA-1 ectodomain in kind, not necessarily in amount. Preferably, said functional part comprises at least part of the prosequence, domain I, domain II and/or domain III of a *Plasmodium falciparum* AMA-1 ectodomain. More preferably, said functional part spans an amino acid sequence corresponding to amino acid residues 25-442, 97-318, 97-442, 97-545, 303-442, 303-544, and/or 419-544 in *Plasmodium falciparum*.

In one embodiment said functional part comprises a subdomain of ectodomain, which can be defined for instance by disulphide bond patterning [7]. By immunogenic property is meant the capability to induce an immune response in a host. Preferably, said immunogenic property comprises a property to induce an immune response against a conformational epitope on a native AMA-1 ectodomain. A functional derivative of a *Plasmodium* AMA-1 ectodomain is defined as a *Plasmodium* AMA-1 ectodomain which has been altered such that at least one immunogenic property of said molecule is essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution. A derivative can also be a fusion of AMA-1 ectodomain or a part thereof with a second protein. In a preferred embodiment said derivative comprises one or more amino acids from variant AMA-1 ectodomains. The resultant AMA-1 ectodomain is a consensus AMA-1 ectodomain having no naturally occurring counterpart. A person skilled in the art is well able to generate analogous compounds of a *Plasmodium* AMA-1 ectodomain. This can for instance be done through screening of a peptide library. Such an analogue comprises at least one immunogenic property of a *Plasmodium* AMA-1 ectodomain in kind, not necessarily in amount. For the present invention complete AMA-1 protein and shorter versions comprising a complete ectodomain are analogous to ectodomain.

Compared to the reported Pf AMA-1 genes, the A+T(U) content of a nucleic acid of the invention is reduced without changing amino acid sequences (with the exception of glycosylation sites, as described below). Preferably said A+T(U) content is reduced in a putative yeast polyadenylation consensus sequence to prevent premature termination of transcription. Such sequences are highly A+T rich and are thus more likely to be present within the A+T rich coding sequences of *P. falciparum* genes. Thus, one embodiment of the invention discloses a method of the invention, wherein at least one putative yeast polyadenylation consensus sequence in said nucleic acid has been modified.

Another problem for expression in eukaryotic systems is N-glycosylation. *P. falciparum* blood stage proteins are not N-glycosylated by the parasite. However, Pf AMA-1 contains 6 N-glycosylation sites that are potentially recognised by other eukaryotic systems. Full length 7G8 Pf AMA-1 expressed in insect cells is glycosylated. Expression of Pv AMA-1 ectodomain in *Pichia* showed heterogeneous glycosylation of the recombinant product [16]. This could only partly be prevented by the addition of extremely high levels of tunicamycin to induction cultures, at the cost of a large drop in expression levels. Deglycosylation using N-glycosidase F was only complete after full denaturation of the protein, a process which would need refolding protocols to obtain properly folded material. Therefore, a preferred embodiment of the invention discloses a method of the invention, wherein at least one site in said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, is removed. Said sites may generally be glycosylated by eukaryotic expression systems through the N-glycosylation pathway. Said site may be removed by mutating the nucleic acid sequence encoding said site. This may lead to a change of at least one amino acid composing said site. Said change may decrease said eukaryotic system's capability of glycosylating said site.

Alternatively, amino acids which are part of said site may be removed without substitution. This may be accomplished by removing a part of the nucleic acid encoding said site. In the present invention it has been found that at least one *Plasmodium* AMA-1 ectodomain potential glycosylation site can be altered to prevent glycosylation at said site in a eukaryotic host, while said altered *Plasmodium* AMA-1 ectodomain retains a capability of raising a cross-reaction immune response in an animal against an unmodified *Plasmodium* AMA-1 protein.

The reasoning for removing a glycosylation site is three-fold. Firstly the presence and location of N-linked glycosylation can have profound but unpredictable targeting and focusing effects on the immune response to proteins [17]. In this context, the Pf AMA-1 baculovirus product had been used in protection studies in Aotus monkeys. These unpublished studies did not show a protective effect of AMA-1 immunisation. Although one explanation for this may have been that a sub-optimal adjuvant was used to formulate the antigen, we reasoned that the glycosylation of the Pf AMA-1 may also have significantly influenced the immune response in a non-beneficial way. Secondly glycosylation is frequently heterogeneous (as demonstrated by expression of the native sequence Pv AMA-1 ectodomain in Pichia). Heterogeneous products may be difficult to reproducibly purify to acceptable standards under GMP, and such heterogeneity may create batch to batch variation in an immunogenic property of the product (given the published effects of N-linked glycosylation on immunogenicity). Thirdly, we wished to produce a protein with the least heterogeneity in order to prepare crystals for crystallographic determination of structure. It is generally accepted that the more homogeneous the protein, the higher the chances of successful crystal formation.

Based on the molecular weight of expressed AMA-1 protein in various

Plasmodium species two groups of *Plasmodium* species can be identified. Those expressing an AMA-1 protein of approximately 66 kDa and those expressing an AMA-1 protein of approximately 83 kDa. A method of the invention is particularly suited to increase levels of expression of ectodomain of the approximately 83 kDa AMA-1 protein in yeast. Measuring the exact molecular weight of a protein is always a difficult task, thus for the present invention the number of 83 kDa should be taken as a guidance for the actual molecular weight of said AMA-1 protein. Variations of 10% in the estimates for molecular weight of a given protein are not abnormal. However, considering the large difference between the two variants of AMA-1 (66 versus 83 kDa) the size indication is only required to help a person skilled in the art determine whether the AMA-1 protein at hand belongs to one or the other class. A variation in the molecular weight measurements of 10% can easily be tolerated while still being able to select one of the two classes of AMA-1 proteins. Thus in a preferred embodiment of the invention said *Plasmodium* belongs to the clade whose members normally express said AMA-1 protein as an approximately 83 kDa protein. "Normally" is herein defined as under conditions occurring in nature. As has already been described in this disclosure, *P. falciparum* and *P. reichenowi* belong to said clade which has the characteristic of expressing said AMA-1 protein as an 83 kDa protein. Another preferred embodiment of the invention discloses a method of the invention, wherein said *Plasmodium* comprises *Plasmodium falciparum*. Preferably, said *Plasmodium* comprises *Plasmodium falciparum* FVO. We have developed the Pf AMA-1 sequence from the FVO strain of *P. falciparum* for expression in *P. pastoris* for several reasons. The challenge strain that is likely to be used in phase II clinical trials is the 3D7 clone of NF54. The FVO strain has an AMA-1 sequence that is one of the most divergent from 3D7 reported to date, and therefore immunisation with FVO AMA-1 would allow for a markedly heterologous challenge. Because of the possibility that polymorphism in AMA-1 is selected and maintained because of immune pressure, the availability of

two extremes of diversity for clinical testing apart and in combination will be extremely informative. In addition, the FVO strain has been adapted to grow in *Aotus lemurinus griseimembra* monkeys, thus allowing preclinical evaluation with homologous challenge possibilities in this non human primate system. FVO as well as 3D7 strains react with mAb 4G2, showing epitope conservation between the divergent AMA-1 sequences.

Another preferred embodiment of the invention discloses a method according to the invention, wherein said yeast is *Pichia*. Yet another preferred embodiment of the invention discloses a method according to the invention, wherein said yeast is *Pichia pastoris*.

In another aspect the present invention discloses an isolated and/or recombinant nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid being modified to utilize a yeast's codon usage. A functional part, derivative and/or analogue of an AMA-1 ectodomain comprises at least one conformational epitope of native AMA-1 ectodomain, preferably said conformational epitope is an ectodomain epitope. Preferably said nucleic acid encodes a *Plasmodium falciparum* AMA-1 ectodomain, more preferably a *Plasmodium falciparum* FVO AMA-1 ectodomain.

As has been described above, preferably at least one putative yeast polyadenylation consensus sequence has been modified in a nucleic acid of the invention. Also, preferably at least one site in said *Plasmodium* AMA-1 ectodomain, or functional part, derivative and/or analogue thereof, that is generally glycosylated by eukaryotic expression systems, is removed. Thus, in a preferred aspect the invention discloses an isolated and/or recombinant nucleic acid sequence according to the invention, wherein at least one putative yeast polyadenylation consensus sequence has been modified. In another preferred aspect the invention discloses an isolated and/or recombinant nucleic

acid sequence according to the invention wherein at least one site in said ectodomain or functional part, derivative and/or analogue thereof that is generally glycosylated by eukaryotic expression systems, is removed.

Figure 1 shows a nucleic acid of the invention, comprising above mentioned preferred characteristics. Thus, in one aspect the present invention discloses an isolated and/or recombinant nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, said nucleic acid comprising a sequence as depicted in figure 1.

Considering that in the present invention a nucleic acid sequence was generated that was modified to utilize a yeast's codon usage and that can be used to express high amounts of *Plasmodium* AMA-1 ectodomain in a yeast cell, and the fact that AMA-1 amino acid sequences of various species comprise significant homology, the present invention further provides a nucleic acid sequence encoding *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, capable of hybridising to at least a functional part of a nucleic acid of the invention. In a preferred embodiment the invention provides a nucleic acid sequence encoding *Plasmodium falciparum* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, capable of hybridising to at least a functional part of a nucleic acid of the invention. Through said hybridisation criterion, it is warranted that said nucleic acid sequence comprises similar expression characteristics (in kind not necessarily in amount) in yeast cells, at least on mRNA level as the nucleic acid of figure 1 which utilizes a yeast's codon usage. By at least a functional part of a nucleic acid of the invention is meant a part of said nucleic acid, at least 30 base pairs long, preferably at least 200 base pairs long, comprising at least one expression characteristic (in kind not necessarily in amount) as a nucleic acid of the invention. Preferably but not necessarily said part comprises an immunogenic property of an AMA-1 ectodomain. In one aspect of

the invention said hybridising to at least a functional part of a nucleic acid of the invention is under stringent conditions.

In another aspect, the invention provides an AMA-1 specific nucleic acid sequence comprising at least 50 percent homology to a nucleic acid sequence of the invention. An AMA-1 specific nucleic acid sequence is defined herein as a nucleic acid sequence, comprising at least 20 nucleotides, preferably at least 50 nucleotides, said sequence comprising a nucleic acid sequence corresponding to at least part of an AMA-1 gene, or comprising a nucleic acid sequence which is complementary to a sequence corresponding to at least part of an AMA-1 gene. In a preferred aspect of the present invention, said AMA-1 specific nucleic acid sequence comprises at least 60 percent homology to a nucleic acid of the invention. More preferably, said AMA-1 specific nucleic acid sequence comprises at least 75 percent homology to a nucleic acid of the invention. In a most preferred aspect of the invention, said AMA-1 specific nucleic acid sequence comprises at least 90 percent homology to a nucleic acid of the invention. Preferably said homology is calculated using the *Plasmodium* AMA-1 ectodomain-specific sequence as depicted in figure 1 as a reference.

With the teachings of the present invention, a person skilled in the art is capable of generating a nucleic acid sequence comprising an immunogenic property of an AMA-1 ectodomain from another species of *Plasmodium*, for instance *Plasmodium vivax* while still using essentially the same nucleic acid sequence as given in figure 1. Such variant nucleic acid will of course still be capable to hybridise to at least a functional part of the nucleic acid depicted in figure 1.

In a preferred embodiment the present invention discloses a nucleic acid sequence according to the invention, wherein said *Plasmodium* belongs to the clade whose members express said AMA-1 protein as an approximately 83 kDa protein. As has been described before, *P. falciparum* and *P. reichenowi* belong to said clade. More preferably, said *Plasmodium* comprises *Plasmodium*

falciparum. More preferably, said Plasmodium comprises *Plasmodium falciparum* FVO.

A nucleic acid of the invention may, for instance, encode a derivative of a *Plasmodium* AMA-1 ectodomain or part thereof, comprising one or more amino acids from variant AMA-1 ectodomains. The resultant AMA-1 ectodomain or part thereof is a consensus AMA-1 ectodomain having no naturally occurring counterpart. Thus, in one aspect the invention provides a nucleic acid sequence according to the invention, wherein said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof comprises a consensus *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof. In a preferred embodiment said part of an AMA-1 ectodomain comprises at least one immunogenic property of said ectodomain. In another aspect, a nucleic acid of the invention may be modified to utilize codon usage of *Pichia*. Thus, in one aspect the invention provides a nucleic acid sequence according to the invention, wherein said yeast is *Pichia*. Preferably, said yeast is *Pichia pastoris*.

A nucleic acid of the invention is particularly suitable for efficient expression of *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof. Therefore, in another aspect the present invention provides a method for producing *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, comprising:
-providing a yeast cell with a nucleic acid according to the invention, and
-collecting formed *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof.

Preferably, said yeast is *Pichia* yeast, more preferably *P. pastoris*. Alternatively, it is possible to express a nucleic acid of the invention in another eukaryotic system, for instance baculovirus or a CHO cell. It is even possible to

express a nucleic acid of the invention in bacteria. Said eukaryotic systems, and bacteria, are more capable of expressing a nucleic acid utilizing yeast's codon usage, compared to a nucleic acid utilizing *P. falciparum*'s codon usage. However, a nucleic acid of the invention can also be modified to utilize codon usage of said other eukaryotic systems, or bacteria. Preferably, said nucleic acid has been modified to remove at least one putative polyadenylation consensus sequence which is recognised by said other eukaryotic system. More preferably, at least one site in said nucleic acid that is generally glycosylated by said other eukaryotic expression system, is removed. Expression of a nucleic acid of the invention in another eukaryotic system, or bacteria, as mentioned above, is still within the scope of the present invention.

Of course, by using a method as previously described, *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue will be produced. Thus, another aspect of the invention provides a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, obtainable by a method of the invention. The invention further provides a Pf AMA-1 ectodomain or functional part, derivative and/or analogue thereof, produced in a yeast cell. In a preferred embodiment said AMA-1 ectodomain or functional part, derivative and/or analogue thereof is purified. As is described in example 4.3, with a method of the invention it is possible to obtain the desired Pf AMA-1 ectodomain, without contaminants like for instance a 50 kDa contaminant. Thus, in a preferred aspect, the invention provides a method according to the invention, further comprising purifying said *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof.

A cell producing said *Plasmodium* AMA-1 ectodomain, or a functional part, derivative and/or analogue thereof, by a method as previously described is of course also within the scope of the present invention. So yet another aspect of the invention provides an isolated cell comprising a nucleic acid of the invention.

In yet another aspect the invention provides an isolated cell comprising a *Plasmodium* AMA-1 ectodomain of the invention or functional part, derivative and/or analogue thereof.

Plasmodium AMA-1 is particularly well suited for the preparation of a vaccine, because accumulated data have indicated that this family of molecules is a target for protective immune responses. As the present invention provides a way of producing a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof efficiently, the invention also provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine. A vaccine comprising a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention and a suitable expedient is of course also herewith provided. Preferably said vaccine comprises a suitable adjuvant.

In a preferred embodiment, at least two different variants of *Plasmodium* AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof according to the invention are used for the preparation of a vaccine. Immunization with different variants of a *Plasmodium* ectodomain or functional part, derivative and/or analogue thereof provides a broader protection. Said vaccine preferably comprises *Plasmodium* AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof from different *Plasmodium* parasites from the same clade. More preferably said vaccine comprises *Plasmodium* AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof from different *Plasmodium* parasites from the same species. Most preferably, said species comprises *Plasmodium falciparum*. A vaccine of the invention preferably comprises *Plasmodium* AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof which display mutual differences, because then a broad protection is even better acquired. An analysis performed by us of *Plasmonium falciparum* variants has revealed

that said variants display mutual differences of between 1 to about 30 amino acid residues. Therefore, a vaccine of the invention preferably comprises *Plasmodium* AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof displaying mutual differences of 1-35, more preferably 15-35, most preferably 25-35 amino acid residues. Said vaccine may for instance comprise *Plasmodium falciparum* FVO Pf83 and *Plasmodium falciparum* 3D7 AMA-1 ectodomains or functional parts, derivatives and/or analogues thereof.

Different variants of a *Plasmodium* ectodomain or functional part, derivative and/or analogue thereof according to the invention can for instance be administered together to an individual at the same time. Alternatively, immunization can be performed with one variant, followed by boosting with another variant. This way, protection against common epitopes is boosted and the formation of parasite invasion inhibitory antibodies which are reactive with a whole range of *Plasmodium* strains is enhanced.

In a preferred embodiment, a vaccine of the invention comprises a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof linked to C3d. Preferably, said C3d is covalently linked. C3d is a complement component that crosslinks receptors on B cells, thus activating them. This results in enhanced antibody production.

In another preferred embodiment the invention provides a vaccine comprising a combination of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof and another *Plasmodium* immunogenic protein or functional part, derivative and/or analogue thereof, like for instance *Plasmodium* MSP1. This way, an even broader protection can be acquired. Said proteins can be present in said vaccine as separate proteins. Alternatively, said proteins can be linked together, or be part of a fusion protein. MSP1, like AMA-1, is involved in the invasion of red blood cells by merozoites. MSP-1 is expressed on the surface of merozoites. Antibodies directed towards the C-terminal end of MSP-1 and reactive with conformational epitopes are capable of blocking invasion *in vitro*.

In yet another aspect the invention provides a use of a proteinaceous molecule capable of binding a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, for the preparation of a vaccine. Said proteinaceous molecule for instance comprises an antibody raised against Pf AMA-1. After administration of said proteinaceous molecule to an individual, said individual is, at least temporarily, protected. Said antibody is preferably a human or humanized antibody. It may be generated *in vitro* using recombinant antibody technology. Alternatively, it may be isolated from blood or serum obtained from an individual vaccinated by a vaccine of the invention. A vaccine comprising a proteinaceous molecule capable of binding a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, and a suitable expedient is therefore also herewith provided. Preferably, a vaccine of the invention is provided wherein said *Plasmodium* comprises *Plasmodium falciparum*. More preferably, said *Plasmodium* comprises *Plasmodium falciparum* FVO.

Of course, a vaccine of the invention is particularly well suited for the prophylaxis of malaria. Thus, the invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine for prevention of malaria.

In a particular embodiment, the invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for the preparation of a vaccine for prevention of malaria, wherein said malaria is caused by *Plasmodium falciparum*. A proteinaceous molecule capable of binding a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof is also well suited for the preparation of a medicament. Preferably, said proteinaceous molecule is used for the preparation of a medicament against malaria.

A *Plasmodium* AMA-1 ectodomain according to the invention is also well

suited for diagnosis of malaria. A person skilled in the art can think of many ways of determining the presence of *Plasmodium* AMA-1 ectodomain, or antibodies against *Plasmodium* AMA-1 ectodomain, in a patient. One way is for instance collecting a blood sample of a patient. Said blood sample can be administered to a well which contains *Plasmodium* AMA-1 ectodomain of the invention. If the patient contains antibodies against *Plasmodium* AMA-1 ectodomain, they will bind to the *Plasmodium* AMA-1 ectodomain in the well. These antibodies can be made visible by many techniques known in the art, for instance by incubation with fluorescent labeled rabbit anti human antibodies. Many other ways are known in the art which are still within the scope of the present invention. Thus, the present invention provides a use of a *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof according to the invention for diagnosis of malaria.

Another embodiment provides a method for, at least in part, diagnosis of malaria, comprising collecting a sample from an individual and providing *Plasmodium* AMA-1 ectodomain or functional part, derivative and/or analogue thereof of the invention with at least part of said sample. Preferably, said sample is a blood sample.

Another embodiment of the present invention provides a method for, at least in part, prophylaxis of malaria, comprising administering a vaccine according to the invention to an individual. Yet another embodiment provides a method for, at least in part, prophylaxis of malaria, comprising administering a proteinaceous molecule capable of binding a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof, to an individual. However, an immune response is often only high directly after administration of a vaccine to an individual. Likewise, protection acquired by administered proteinaceous molecules capable of binding a *Plasmodium* AMA-1 ectodomain or a functional part, derivative and/or analogue thereof is often only high directly after administration of said proteinaceous molecules to an

individual. Therefore, a preferred embodiment of the present invention provides a method for, at least in part, prophylaxis of malaria comprising administering to an individual slow release compositions comprising a vaccine of the invention.

By slow release composition is meant a composition from which a vaccine of the invention is only slowly migrated into the body. This way, said body contains a vaccine of the invention for a prolonged period, so the immune-response will be high during a prolonged period of time.

The following examples illustrate the present invention. The examples do not limit the present invention in any way. A person skilled in the art can perform alternative ways which are still in the scope of the present invention.

EXAMPLES

1 Development of synthetic gene for *P. falciparum* FVO strain Pf AMA-1.

1.1 Original FVO sequence

Cryopreserved parasite stocks from *P. falciparum* FVO were prepared from an infected *Aotus lemurinus griseimembra* monkey at the young ring stage of development and DNA was isolated (Gentra systems Inc., Minneapolis, MN) directly from a parasite stock according to the manufacturer's instructions. Pf AMA-1 was amplified by polymerase chain reaction using *Pfu* polymerase (Promega, Leiden, The Netherlands) and primers PF83A: 5'-GGGGGATCCATGAGAAAATTATACTGCGTATT-3' (nt 1-23 and additional *Bam*HI restriction site) and PF83B: 5'-ACGTGGATCCTTAATAGTAT-GGTTTTTCCATCAGAACTGG-3' (complementary to nt 1843-1869 and additional *Bam*HI restriction site) containing *Bam*HI restriction sites to facilitate cloning in pBluescript. A pool of four independent clones was used for sequence analysis using an ABI Prism™ 310 automated sequencer (PE Applied Biosystems, Foster City, CA) according to the manufacturers instructions, and primers previously synthesised for sequencing of Pf AMA-1 [10]. This resulted in the unambiguous sequence of *P. falciparum* FVO Pf AMA-1, that differs from the FVO AMA-1 sequence available from Genbank (accesssion number U84348) at three amino acid positions. The most notable difference is that the Genbank FVO AMA-1 sequence is one amino acid shorter than any other available AMA-1 sequence, and our FVO AMA-1 sequence does not have this deletion.

1.2 Alteration of N-glycosylation sites

The sequence of gene Pf AMA-1 from FVO strain that we have established encodes a protein of 622 amino acid residues that has 6 potential N-glycosylation sites. Our previous experience with expressing Pf AMA-1 in

baculovirus/insect cells as well as with expressing Pv AMA-1 in *P. pastoris* has shown that these N-glycosylation sites will be glycosylated in eukaryotic heterologous expression systems. As explained above, this is undesirable since native Pf AMA-1 is not glycosylated. Therefore we developed a variant that exploited the lack of conservation of N-glycosylation sites in published *Plasmodium* AMA-1 allele sequences. Asn 162 was changed to Lys that is present in that position in Thai-Tn strain Pf AMA-1 (accession nr M58547). Thr 288 was changed to Val (present in *P. vivax* and *P. knowlesi* AMA-1; accession nrs Y16950 and M61097); Ser 373 was changed to Asp (present in *P. knowlesi* AMA-1); Asn 422 and Ser 423 were changed to Asp and Lys, respectively (present in *P. knowlesi, P. vivax, P. chabaudi* (accession nr M25248) and *P. fragile* AMA-1(accession nr M29898)) and Asn 499 was changed to Glu (present in *P. chabaudi* AMA-1).

1.3 Synthetic gene with P. pastoris codon usage

The nucleotide sequence with the six changed codons to delete the potential N-glycosylation sites was used to develop a synthetic gene utilising the codon usage of *P. pastoris* (NIMR, London). Our previous experience with expressing Pf AMA-1 in *P. pastoris* taught us that the high A+T content of the *P. falciparum* gene makes it extremely difficult to express this in *P. pastoris*. There are several A+T rich regions within the coding sequence that are recognised as transcription termination and/or polyadenylation sites in yeast, resulting in truncated mRNAs and no protein production. The sequence of the synthetic gene was designed according to *P. pastoris* codon usage with the aid of the CODOP program [18]. This program allows codon optimisation with host organism preference. It enabled design of an optimal sequence, with strategic insertion of restriction sites, and the generation of oligos of 40 nucleotides in length from both strands of the gene. The resulting set of 92 oligos was rigorously screened for the presence of potential transcription termination signals and undesirable repeats, inverted repeats, and regions of

complementarity which could potentially lead to nonspecific intermolecular hybridisation. The 20 nucleotide overlap between each 40-mer primer was adjusted to give a melting temperature in the range 68-62 °C, in order to allow subsequent use of the primers for DNA sequencing. Gene synthesis was by assembly polymerase chain reaction (PCR), using the proof-reading *Pfu* DNA polymerase, as described in reference [18]. Blunt-ended PCR products corresponding to each 'half' of the gene were cloned into *pMos*Blue (Amersham Pharmacia) and fully sequenced on both strands before subcloning to produce the complete synthetic gene. The final product was again sequenced on both strands. The sequence of the synthetic gene FVO Pf83syn is provided in figure 1.

2 Expression of FVO Pf83syn ectodomain in *P. pastoris*

2.1 Development of expression constructs

For secreted expression in *P. pastoris* strain KM71H we used vector pPICZαA (Invitrogen). This vector provides an N-terminal signal sequence and a C-terminal myc epitope followed by a 6 x His tag for easy purification. Gene fragments have to be cloned in frame with these sequences. Primers for PCR amplification of the Pf AMA-1 ectodomain were Pf83A: 5'-GGAATTCCAGAACTACTGGGAGCATCC-3' (nt 73-92 and additional *Eco*RI restriction site) and Pf83H: 5'-GCTCTAGAATGTTATCGTAACGTAGGCTT-3' (complementary to nt 1615-1634 and additional *Xba*I restriction site) or Pf83A and Pf83I: 5'-GCTCTAGACTACATGTTATCGTACGTAGGCTT-3' (complementary to nt 1615-1635, plus stopcodon plus additional *Xba*I restriction site; this provides the full ectodomain without myc epitope and His tag). A 50 µL PCR reaction contained 10 ng template DNA (FVO Pf83syn), 100 ng of each of the primers Pf83A and Pf83H, or Pf83A and Pf83I, 0.2 mM dNTP, 5 µL 10x *Pfu* reaction buffer and 1 unit *Pfu* polymerase (Promega). Amplification proceeded as follows: 1 min, 94°C, 1 min 52°C, 1.5 min 72°C for 3

cycles; 1 min, 94°C, 1 min 60°C, 1.5 min 72°C for 30 cycles; 5 min, 72°C and then stored at 4°C. The resulting 1578 bp PCR product was digested with *Eco*RI and *Xba*I sequentially, and ligated into *Eco*RI/*Xba*I digested pPICZαA in a 1:10 molar ratio. *E. coli* DH5α subcloning efficiency cells were transformed with 5 μL of the ligation mixture and plated on low salt LB plates containing 25 μg/ml zeocin and cultured overnight at 37°C. Colonies were grown in low salt LB containing 25 μg/ml zeocin, plasmids were isolated by standard miniprep methods and analysed by restriction enzyme digestion. One clone containing the correct insertion for each of the PCR products (named Pf4mH for primers A and H, and Pf11-0 for primers A and I) was used to isolate plasmid DNA for transformation of *P. pastoris*.

2.2 Pichia transformation and analysis

The expression construct was linearised with *Sst*I and 10 μg DNA was used to transform 80 μL *P. pastoris* KM71H cells by electroporation following the Invitrogen protocols. 1 ml of 1M sorbitol was added and the cells were allowed to recover for 2h at 30°C. Cells were then plated (25, 50, 100, 200 μL aliquots) on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) agar plates containing 100 μg/ml zeocin, and incubated for 4 days at 30°C. Colonies were picked and grown for 2 days at 30°C in 10 ml of BMGY (1% yeast extract, 2% peptone, 1.34% Yeast Nitrogen Base, 1% glycerol, 0.4 mg/L biotin, 0.1M K-phosphate pH 6.0) in 50 ml Falcon tubes with vigorous shaking. Cells were harvested by low-speed centrifugation, resuspended in 4 ml of BMMY (BMGY with glycerol substituted for 0.5% methanol), and cultured for an additional 2 days. Cells were harvested and the culture supernatants were analysed for the presence of Pf AMA-1 ectodomain by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue. All clones analysed expressed an equal amount of two proteins in the culture supernatant. A 50 kDa molecule of thus far unknown origin as well as an approximately 75 kDa protein, which proved to be the Pf AMA-1 ectodomain, with or without myc epitope and His tag

(Pf4mH and Pf11-0, respectively). Expression levels in these small scale cultures are estimated to be 50 mg/L. Our experience with the expression of Pv AMA-1 in *P. pastoris* suggests that this might result in levels approaching 1 g/L in optimised fermentations. No obvious degradation products were visible in the culture supernatants.

Culture supernatants of Pf4mH were spot blotted on nitrocellulose membranes and incubated with rat monoclonal antibody 58F8 (recognising a linear epitope in the N-terminal region of Pf AMA-1), or 4G2 (recognising a conformational epitope in the ectodomain and capable of blocking parasite multiplication *in vitro*) for 1 h at room temperature. After incubation with goat-anti-rat IgG, colour was developed using NBT/BCIP. Only culture supernatants from the recombinant *P. pastoris* expressing the 75 kDa protein reacted with both mAbs. Control culture supernatants, where the 50 kDa protein, but not the 75 kDa protein, was present did not react with either of the mAbs. This indicates that the 75 kDa protein is the Pf AMA-1 ectodomain and that the secreted material is properly folded. As expected, reactivity with 4G2 was lost when the culture supernatant was reduced with β-mercaptoethanol prior to spot blotting, demonstrating the correct disulfide bond formation within the ectodomain to recreate the 4G2 epitope.

Purified Pf4mH (sec 4) was used in a standard ELISA to test reactivity with mAb 4G2 and a human serum from an African endemic region. These human sera show high reactivity with conformational epitopes of AMA-1, and hardly react with reduced AMA-1. In this ELISA, strong reactivity with 4G2 and the human serum was detected, whereas a control mAb and a pool of European human serum did not react. As a positive control, similar amounts of baculovirus produced Pf AMA-1 were coated on an ELISA plate and incubated with the same serum samples. Similar results were obtained, although reactivity was much lower, suggesting a much better quality for the *Pichia* Pf4mH product.

In addition, rabbit sera raised against the baculovirus produced Pf AMA-1 displayed much lower titers on Pf4mH than rabbit sera raised against Pf4mH. This was not due to impurities in the Pf4mH preparation, since: 1) a very low reactivity of the anti-Pf4mH sera against *Pichia* proteins was observed, and 2) anti-*Pichia* antisera were only marginally reactive with contaminations in purified Pf4mH by Westernblotting. These results indicate that the baculovirus produced Pf AMA-1 is less immunogenic, most likely due to the relative impurity of the purified product and/or heterogeneity in folding of the product.

The homogeneity of the *Pichia* produced Pf4mH was further evaluated by immuno-affinity chromatography, using immobilised mAb 4G2, reactive with a conformational epitope. It was found that Pf4mH quantitatively bound to the immobilised mAb, demonstrating that every molecule has the proper conformation.

To determine an Pf AMA-1 epitope for mAb 4G2, we expressed separate domains of Pf AMA-1 and combinations thereof using the same *P. pastoris* system as for Pf4mH. These are:
Pf3mH: amino acid residues 25-442 (prosequence, domains I and II);
Pf8mH: amino acid residues 303-442 (domain II);
Pf9mH: amino acid residues 303-544 (domains II and III);
Pf10mH: residues 419-544 (domain III); and
Pf14-0: residues 97-545 (domains I, II, III).
Residue 97 is the natural N-terminus of the 66 kDa proteolytic product of the 83 kDa Pf AMA-1 [21]. We established that the parasite-inhibitory mAb 4G2 is only reactive with Pf3mH, Pf4mH and Pf14-0, and not with any of the other proteins. This maps an epitope for 4G2 to domain I or domains I + II.

Immunogenicity has been evaluated in rabbits by 4 immunizations of 100 microgram protein formulated in Freunds complete (1st injection) or Freunds incomplete (remaining injections) adjuvant. Injections were given at

days 0, 14, 28 and 56, and antisera obtained 4 weeks after the final boost were tested by ELISA and immunofluorescence (IFA). Results for Pf4mH are summarized in Table 1 and IFA data from the other rabbit sera are summarized in Table 2. It is clear that all AMA-1 domains produced by us are capable of inducing high levels of antibodies that are reactive with the native parasite protein. Using the same protocol, the immunogenicity of two additional fragments are evaluated. These fragments comprise:

1) amino acid residues 97-442 (domain I + II), and

2) amino acid residues 97-318 (domain I).

IgG was purified from immunized rabbits using standard procedures and the capacity to inhibit *P. falciparum* growth *in vitro* was evaluated. Parasites at mature schizont stage were cultured in 96-well plates in the presence of different concentrations of IgG from the immunized rabbits, or of IgG from control rabbits immunized with adjuvant only, or purified mAb 4G2 IgG. Radiolabel was added after re-invasion of erythrocytes had occurred (approx. 17 h later) and *in vitro* culture was continued for another 10 h. Parasites were harvested onto glass fiber filters using a Titertek cell harvester (ICN). Incorporation of [^{3}H]hypoxanthine was determined by liquid scintillation spectrometry. Parasite growth inhibition, reported as a percentage, was determined as follows: 100 - ((average $CPM_{experimental}$/average $CPM_{control}$) x 100). The incorporation for erythrocytes alone was subtracted from all averages prior to determining the percentage inhibition. Control IgG was isolated from rabbits that had been immunized with adjuvant only.

In this assay, mAb 4G2 at 1 mg/ml gives 50-60% inhibition of invasion, irrespective of the *P. falciparum* strain used. Data for the Pf4mH-immunised rabbit IgGs are given in Table 1. We used FCR3 as the homologous strain, since AMA-1 differs by only 1 amino acid residue, located in the pro-sequence, from FVO AMA-1. NF54 was used as the heterologous strain and differs by 29

amino acids from FVO AMA-1. Total IgG from rabbits immunized with Pf4mH inhibit invasion of the homologous strain up to 85% at 1.5 mg/ml (a concentration far below standard serum IgG concentrations), and of the heterologous strain up to 58%. This indicates the presence of common as well as strain-specific epitopes and demonstrates the capacity of the *Pichia* produced Pf AMA-1 ectodomain to induce potent parasite-inhibitory antibodies.

Table 1. Analysis of anti-Pf4mH responses

Rabbit	ELISA titer		IFA titer		Inhibition of invasion[1]	
	Pf4mH	pPICZα	FCR3	NF54	FCR3	NF54
1	2.5×10^6	4×10^4	2.5×10^5	1.3×10^5	85%	55%
2	2.5×10^6	4×10^4	2.5×10^5	0.6×10^5	75%	58%
3	1.3×10^6	$<1 \times 10^4$	1.3×10^5	0.3×10^5	50%	44%

[1] evaluated using purified IgG at 1.5 $mg.ml^{-1}$

Table 2 Immunogenicity of AMA-1 domains

Rabbit ID	Antigen	AMA-1 residues	IFA titer
			FCR3
715	Pf11-0	25-545	2.5×10^5
716			5.1×10^5
717			2.5×10^5
709	Pf3mH	25-442	2.5×10^5
710			2.5×10^5
771	Pf8mH	303-442	1.3×10^5
772			2.5×10^5
773	Pf9mH	303-544	1.3×10^5
774			1.3×10^5
775	Pf10mH	419-544	0.3×10^5
776			1.3×10^5

3 Bulk production

Pf11-0.1 has undergone a feasibility study for GMP production at a GMP production facility. Pilot fermentations at 5-10 L scale have been performed to assess parameters that influence proteolytic degradation and yield. The conclusion was that addition of 0.4 mM EDTA to the standard fermentation medium at pH6.0, as well as methanol induction with a high cell density for a short period of 30 h, and immediate freezing of the harvested culture supernatant until processing are all beneficial to prevent proteolytic degradation. For purification best results were obtained by direct binding of Pf AMA-1 on an immobilized metal affinity column activated with $CuSO_4$ (IMAC). This step also removes proteases from Pf AMA-1 resulting in an increase in stability of the partially purified product. The general conclusion of the feasibility study is that it is feasible to produce 1 gram of protein with a minimum purity of 98% for Phase I clinical testing.

For mid-scale production of Pf AMA-1 ectodomain recombinant *P. pastoris* was cultured in 1L baffled flasks (400 ml BMGY per flask) for 48 h at 29-30°C under vigorous shaking. Cells were harvested and resuspended in 100 ml BMMY, and then cultured for 48 h at 29-30°C under vigorous shaking. Methanol was added to a final concentration of 0.5% every 24 h. After low-speed centrifugation, the culture supernatant was harvested. Protein was precipitated with amonium sulphate (70% final concentration) at 0°C, and the precipitate was stored at 4°C until use.

4 Purification strategies

4.1 Purification of Pf4mH on Ni resins

Additional proof that the secreted 75 kDa protein is the Pf AMA-1 ectodomain comes from purification using Ni resins, since recombinant proteins produced using the pPICZα vector contain His tags that have a high affinity for Ni. The ammonium sulphate precipitate of 50 ml culture

supernatant was solubilised in 2 ml binding buffer (20 mM Na Phosphate pH 7.8, 0.5 M NaCl) and loaded on an 8 ml Ni-agarose column (Probond, Bio-Rad) at 0.2 ml/min. The column was washed at 1 ml/min with 15 ml binding buffer, 25 ml of the same buffer pH 6.0, 15 ml of the buffer pH 5.5 and then eluted with the same buffer at pH 4.0. Elution was monitored at 280 nm. The pH 4.0 peak fractions contained a single protein of 75 kDa as determined by SDS-PAGE analysis. Alternatively, the 75 kDa ectodomain could be eluted with a linear 0-500 mM Imidazole gradient in 20 mM Na Phosphate pH 6.0, 0.5 M NaCl. Spot blotting of the peak fractions revealed strong 4G2 and 58F8 binding, indicating that the 75 kDa protein is the His-tagged Pf AMA-1 ectodomain. The 50 kDa protein present in the culture supernatant as well as yellow-stained flavin components were present in the flow through and pH 6.0 wash fractions.

4.2 Other purification strategies for Pf11-0

Other purification strategies are needed when the ectodomain is expressed without His tag, which might be more appropriate for clinical purposes. One way of purifying the 75 kDa ectodomain Pf11-0 away from the 50 kDa protein is the use of hydroxy apatite (HAP) [19] [20] chromatography.

The ammonium sulphate precipitate of 100 ml culture supernatant was solubilised in 5 ml 10 mM $NaPO_4$, pH 6.8 and loaded onto a prepacked 5 ml HAP column (CHT-II, Bio-Rad) at 0.5 ml/min. Elution with a 20 ml gradient to 400 mM $NaPO_4$, pH 6.8 at 1 ml/min was monitored at 280 nm. Two overlapping peaks were evident, the first one containing mainly the 50 kDa protein, the second one mainly the Pf AMA-1 ectodomain. Further purification could be obtained by subsequent anion exchange chromatography of the pooled second peak fractions after diluting 1:10 in miliQ water on a prepacked 5 ml UNO Q column (Bio-Rad), eluted with a linear gradient of 0-0.5 M NaCl in 20 mM Tris.HCl pH 7.6. This results in several peaks containing the remainder of the 50 kDa contaminant as well as several degradation products of the AMA-1

ectodomain, and a single peak that contains pure intact AMA-1 ectodomain, as analysed by reduced SDS-PAGE and Coomassie staining.

4.3 *Production of Pf11-0 without the contaminating 50 kDa protein*

The 50 kDa protein present in the culture supernatant of our recombinant *P. pastoris* KM71H clones is not common (information from Invitrogen). Transformation of just the empty pPICZα vector into the same batch of *P. pastoris* KM71H also yielded a 50 kDa protein in the culture supernatant upon methanol induction. Untransformed *P pastoris* KM71H does not produce this protein. We have now succeeded in preparing a new clone (Pf11-0.1) that only secretes the 75 kDa Pf AMA-1 ectodomain upon methanol induction, and that does not produce the 50 kDa contaminant. This was achieved by picking a single colony of *P. pastoris* KM71H from a freshly prepared agar plate, made from the original stock of that strain. This colony was used to start fresh cultures, that were transformed with the Pf11-0 vector, resulting in the above described expression.

Purification as described under 4.2 will provide higher yields of pure Pf AMA-1 ectodomain, since there is no need to separate the 75 kDa product from a major contaminant any more, allowing to take the complete peak fraction from the HAP column for further anion exchange chromatography purification.

Brief description of the drawings

Figure 1: sequence of an isolated and/or recombinant nucleic acid of the invention, encoding *Plasmodium* AMA-1 ectodomain. Surprisingly, this sequence is very well expressed in *Pichia pastoris*, whereas a nucleic acid sequence encoding wild-type Pf AMA-1 ectodomain is not.

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